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Research Article

Metabolism, movement behaviour, and salinity responses of invasive mosquitofish compared with a threatened toothcarp

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Abstract

The success and impact of invasive non-native species depend on how they cope with local abiotic conditions, especially in comparison to co-occurring native taxa. In aquatic systems, salinity serves as a key environmental filter, mediating the establishment of invasive species and influencing biotic interactions. However, the mechanistic basis behind these context-dependent responses remains poorly understood. In this study, we examined interspecific differences in metabolic rate, movement behaviour, and their relationship between the worldwide invasive *Gambusia holbrooki* and a threatened Spanish endemic fish *Aphanius iberus* across an experimentally-manipulated salinity gradient. Using intermittent-flow respirometry and automated tracking of movement patterns, we compared aerobic metabolic traits and movement behaviours under various salinities. Toothcarp maintained a stable aerobic scope across salinity treatments, whereas invasive mosquitofish exhibited a markedly lower aerobic scope with higher salinity, primarily due to a decline in maximum metabolic rate. This stress response was not linked to increased osmoregulatory costs, as baseline metabolism decreased. Behavioural tests demonstrated consistent species differences in routine locomotion, with mosquitofish showing more exploratory behaviour and toothcarp showing more stop-go behaviour and remaining more stationary overall. Notably, in toothcarps, we found a negative link between standard metabolic rate and space use, suggesting that individuals with higher baseline metabolism may be constrained in their movement. Conversely, in mosquitofish, although salinity affected metabolic capacities, this effect was not reflected in their movement, indicating a weak relationship between metabolism and behaviour, likely supported by trait flexibility. By integrating metabolic traits with behavioural data, our results reveal mechanisms underlying invasive species responses and strengthen predictions of their performance relative to native fishes under changing conditions, such as salinity in inland waters. This highlights the importance of trait-based approaches for predicting responses to abiotic stressors and for evaluating the ecological impacts of invasive taxa.

Key words: Aerobic scope, *Aphanius iberus*, context-dependent competition, *Gambusia holbrooki*, intermittent-flow respirometry, invasive non-native species, movement behaviour

Introduction

Invasive non-native species (INS) are a major driver of biodiversity loss worldwide, often outcompeting native species and disrupting ecosystem functions (Ricciardi et al. 2013; Seebens et al. 2017). Their success frequently depends on how they

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respond to local abiotic conditions, particularly in contrast to native species that have evolved under those same environments (Alcaraz et al. 2008a; Vivó-Pons et al. 2024). As a result, the ecological impacts of INS are highly context-dependent, varying not only across ecosystems but also along environmental gradients such as temperature, oxygen, and salinity (Dunson and Travis 1991; Rahel and Olden 2008; Ricciardi et al. 2013). In many aquatic ecosystems, salinity stands out as a key abiotic filter, acting as a physiological barrier that limits dispersal and influences INS establishment (Bœuf and Payan 2001; Cañedo-Argüelles et al. 2013). These effects are particularly relevant in estuaries and coastal wetlands, where strong salinity gradients significantly influence interactions between native and invasive species (Scott et al. 2008; Newton et al. 2020).

For aquatic animals, the primary impact of salinity arises from the physiological costs of maintaining osmotic balance through active regulation (Ern et al. 2014; Anufriieva and Shadrin 2023). These costs lower aerobic scope, limiting the energy available for movement, growth, and reproduction (Ern et al. 2014; Farrell 2016; Pang et al. 2020). Within an ecophysiological perspective, salinity stress may help explain the limited success of INS relative to native taxa (Behrens et al. 2017; Backström and Winkelmann 2022; Boardman et al. 2022) because evolutionary history shapes the salinity range over which species maintain osmotic comfort, which in turn can influence invasion outcomes (Freire 2025). For instance, Backström and Winkelmann (2022) found that the invasive round goby (*Neogobius melanostomus*) showed reduced metabolic performance under elevated salinity compared to the native European perch (*Perca fluviatilis*).

Because energy availability is essential for movement, salinity stress can impair behaviours related to dispersal, foraging, and predator avoidance (Pang et al. 2020). INS often exhibit high activity, boldness, and exploratory tendencies, which support their spread (Rehage and Sih 2004; Rehage et al. 2005; Damas-Moreira et al. 2019). However, these advantages may be constrained by salinity, which can reduce mobility and foraging through higher osmoregulatory costs than those of native competitors (Alcaraz et al. 2008a; Pirroni et al. 2024). Thus, the physiological impact of abiotic stressors can hinder movement patterns, and metabolic responses may limit behavioural flexibility and space use under stress (Biro and Stamps 2010; Killen et al. 2013; Baktoft et al. 2016). Integrating metabolic and behavioural traits is key to understanding and predicting the outcomes of invasion dynamics (Myles-Gonzalez et al. 2015; Christensen et al. 2021; Schneider et al. 2024), as behaviour forms an integral part of physiological function and cannot be fully understood in isolation from underlying metabolic processes (Debaere et al. 2024). Surprisingly, empirical studies addressing this integration in the context of salinity stress remain scarce (e.g. Backström and Winkelmann 2022).

Coastal wetland species offer an ideal system to investigate these dynamics, as salinity gradients in these habitats strongly influence interactions between INS and native species. A well-documented example is the case of the eastern mosquitofish (*Gambusia holbrooki*), one of the 100 worst invasive species (Lowe et al. 2000), and the Spanish toothcarp (*Aphanius iberus*), an endemic fish threatened by INS and habitat destruction. The toothcarp now persists only in a few isolated Mediterranean coastal wetlands populations along the eastern Spanish coastline (Oliva-Paterna et al. 2006). Mosquitofish thrive in freshwater and brackish habitats due to rapid reproduction, broad physiological tolerance, and aggressive

behaviour (Pyke 2005; Ruiz-Navarro et al. 2011). In many lowland systems, mosquitofish have displaced toothcarp, which largely remain in high-salinity habitats where mosquitofish seem to be less competitive (Rincón et al. 2002; Alcaraz et al. 2008a, 2008b; Ruiz-Navarro et al. 2011, 2013). Some studies suggest that elevated salinity may also hinder toothcarp metabolism and impair growth, although this has not been directly tested (Sgarzi et al. 2020). In fresh waters, toothcarp exhibit slightly higher metabolic rates, possibly reflecting greater activity (Rubio-Gracia et al. 2020). However, they exhibit small home ranges and low swimming performance (Alcaraz et al. 2008b; Gonzalez et al. 2018; Rubio-Gracia et al. 2020). This highlights the importance of using a trait-based approach that considers both physiology and movement behaviours together to understand species coexistence across salinity gradients.

In this study, we investigate how metabolic traits, movement tendencies, and their interplay differ between mosquitofish and toothcarp across an experimental salinity gradient. Specifically, we quantified aerobic metabolism (standard metabolic rate, SMR; maximum metabolic rate, MMR; and aerobic scope) and movement tendencies (movement speed, exploration, and stop-go behaviour) across freshwater and moderate salinities. We hypothesised that mosquitofish would exhibit reduced metabolic capacity (higher SMR, lower MMR, and reduced aerobic scope) at elevated salinity, while toothcarp would maintain more stable performance. Moreover, we expected mosquitofish to be more active and exploratory overall but to reduce movement with increased salinity, whereas toothcarp movement would remain more consistent. By integrating physiological and behavioural data, we aim to clarify the mechanisms that influence species coexistence in salinity-structured ecosystems.

Methods

Fish collection and holding

Spanish toothcarp (*Aphanius iberus*) and eastern mosquitofish (*Gambusia holbrooki*), hereafter referred to as "toothcarp" and "mosquitofish" for simplicity, were collected using minnow traps in February 2021 from the La Pletera saltmarshes in Baix Empordà, northeastern Spain (42°01.82'N, 3°11.48'E). Afterwards, to increase the sample size, a second sample of toothcarp was collected from the Rubina saltmarshes in Alt Empordà (42°14.80'N, 3°07.00'E). Salinity was measured at the same depth in both lagoons using a multiparameter probe (HI98194, Hanna Instruments, Woonsocket, RI, USA), yielding a value of 15 on the practical salinity scale at the time of capture. Note that throughout this study, all salinity values are reported according to the Practical Salinity Scale (i.e. unitless), as recommended by UNESCO (1985), and were derived from conductivity measurements. After sampling, fish were immediately transported to the laboratory, where they were housed in separate aquaria based on species, sex, and salinity treatment (see below). On the first day, fish were transferred to the aquaria with water from the sample site, and this water was gradually replaced with water from the lab at the correct temperature and salinity. The aquaria (60 L) were equipped with biological filters, an aeration system, and artificial plants. The water temperature was gradually decreased and maintained at 20 ± 0.5 °C, and the salinity was maintained at 15. Lights were on an automated photoperiod of 12 h:12 h. From the second day of acclimation, the fish were fed once a day with frozen bloodworms (*Chironomus* sp.)

ad libitum. Two weeks after collection, we initiated the process of changing salinity to generate the different salinity treatments (0.26 (freshwater), 15, 25, and 40) by adding artificial sea salt to increase salinity or adding water to decrease salinity, until the required salinity was achieved.

Experimental overview

The experiment was conducted in two phases. The first phase involved a mixed sample of 37 mosquitofish (body mass: 0.36 ± 0.01 g (mean ± SE); range: 0.13–0.64; Suppl. material 1) and 60 toothcarp (body mass: 0.37 ± 0.02 g; range: 0.19–0.68; Suppl. material 1) collected from La Pletera, with experiments running from May to July. The second phase used only 90 toothcarp (body mass: 0.37 g, mean ± 0.01 SE; range: 0.19–0.69 g; Suppl. material 1) from the Rubina population, with trials conducted from late September to mid-October. Acclimatisation to target salinity treatments was the same for both groups. Once the specific treatment salinity was reached, fish were left to acclimatise for two weeks prior to testing. Following acclimatisation, each fish was individually tested with a 24-hour respirometry trial, after which they were transferred to individual holding tanks to facilitate identification. The holding tanks were custom-built acrylic setups consisting of two rows of six compartments (each 10 cm wide and 20 cm long), separated by transparent, perforated partitions that allowed visual and olfactory cues to pass through. Fish remained in the holding tanks for one day before being tested in behavioural trials. To standardise hunger levels and minimise postprandial effects, fish were fasted for 24 hours prior to all trials.

Respirometry experiments

To measure the oxygen consumption rate ($\dot{M}O_2$) of each fish, we used an intermittent flow respirometry system (Loligo® Systems, Tjele, Denmark). The system included four cylindrical glass chambers (18.5 mm internal diameter × 40 mm length; 10.7 ml volume) that provided a chamber volume-to-fish mass ratio of approximately 20:1. The chambers were arranged in a holder and submerged in water in a transparent acrylic bath (400 mm length × 225 mm width × 140 mm height). The system was connected to an external 300-litre tank, from which a pump (2400 L h^{-1}, Eheim Universal Pump 2400) continuously provided air-saturated water to the bath and recycled the excess water through a decantation system. The temperature was maintained at 20 ± 0.5 °C using an automated liquid cooler (972.46 BTU/h, J.P. Selecta). Moreover, the bath had two miniature DC pump systems attached, one for flushing at a flow rate of approximately 100 mL/min and one for recirculating (mixing) water within the chambers at a flow rate of 90 mL/min. Thus, minimising the oxygen stratification within the chamber (Rodgers et al. 2016; Svendsen et al. 2016; Killen et al. 2021). Oxygen measurements were taken using non-invasive sensor spots attached to the interior of the chambers. Four fibre-optic probes, aligned with these sensor spots, were connected to a Witrox-4 oxygen meter, which recorded oxygen levels at 0.5 Hz using AutoResp v2 software (Loligo Systems, Tjele, Denmark). Between each trial, sensors were calibrated to each salinity treatment and temperature using a 2-point calibration method, which requires two measurements: one at 100% air saturation and the other at 0% air saturation, achieved by adding sodium sulphite (Na_2SO_3, 0.159 M).

At the start of the trial, fish were weighed (± 0.01 g) and placed in a circular exercise area (230 mm in diameter, 120 mm in water depth) filled with aerated water at the appropriate salinity. After one minute of acclimation, fish were chased by hand to induce exhaustion (Norin and Clark 2016; Andersson et al. 2020). This was done by agitating the water near the tail of the fish by hand and gently tail-tapping to encourage burst swimming (Norin and Clark 2016; Andersson et al. 2020). Finally, once the fish was visibly exhausted, it was promptly transferred to the respirometry chambers. In the respirometer, measurement cycles had three phases: a 360-second measuring phase, a 300-second flush phase, and a 120-second wait phase, totalling 780 seconds per cycle. Oxygen consumption was recorded during the measuring phase, beginning approximately 40 seconds after the chase and continuing for one hour. Fish remained in the chambers overnight for a total monitoring period of approximately 23 hours. Further methodological details of the intermittent-flow respirometry setup are provided in Suppl. material 2, following the reporting standards recommended by Killen et al. (2021). Sample sizes differed across salinity treatments due to natural variation in sex ratios, daily processing limits, and the inability to test mosquitofish at the highest salinity. For the three treatments that included both species, sample sizes were as follows: at 0.26, we tested 28 toothcarp females, 17 toothcarp males, and 14 mosquitofish females; at 15, we tested 29 toothcarp females, 10 toothcarp males, and 14 mosquitofish females; and at 25, we tested 8 toothcarp females, 10 toothcarp males, and 9 mosquitofish females. At the highest salinity (40), only toothcarp were available, with 29 females and 19 males tested.

Behavioural experiments

A day after being tested in the respirometry experiments, the fish were individually analysed in one of three identical square arenas (60 × 60 cm), made of rigid, thick green PVC sheet (water height: 10 cm). The tanks were positioned in a covered structure to reduce external disturbances and provide diffused light from above. Raspberry Pi cameras in the top of the structure, directly above each tank, were used to film the trials at 20 fps using pirecorder software (Jolles 2020). The tanks contained sand as substrate, stone pebbles for structure, and two patches of artificial plants on opposite sides of the tank as refuge, each with a diameter of 15 cm (Suppl. material 3: fig. S1). Water in the tanks was maintained at the salinity corresponding to the holding treatment and was carefully checked before the start of the trials. The water in the tanks was continuously aerated, except during the experimental trials.

At the start of an experimental day, fish were removed from their individual holding compartments according to a randomised schedule and transferred to a transparent Perspex acclimatisation cylinder located in the centre of the tank. Subsequently, the curtains around the structure were carefully lowered, and the fish given one minute of acclimation, after which the cylinders were remotely lifted. After an additional four minutes of acclimation, video recording was automatically started and continued for a total of 15 minutes. After the trials, we lifted the curtain around the tanks, removed the fish using a fishnet, briefly placed them one after another on a wetted measuring board, and measured their body length to the nearest millimetre, after which they were returned to their holding compartments. Behavioural trials were conducted on a subsample of individuals drawn randomly

from those used in the metabolic experiment, but only for the two lowest salinity treatments (0.26 and 15). At salinity 0.26, the subsample included 10 toothcarp females, 9 toothcarp males, and 11 mosquitofish females. At salinity 15, we tested 9 toothcarp females, 9 toothcarp males, and 9 mosquitofish females.

Data processing

Raw data from Loligo® Systems software were processed in Excel. $\dot{M}O_2$ was calculated from the slopes of linear regressions of oxygen concentration over time within each measurement cycle, adjusted for respirometer volume. To calculate the MMR, we recalculated slopes for the initial 3-minute post-chase period, as oxygen consumption usually peaks during this time (Norin and Clark 2016). SMR was determined by averaging the ten lowest $\dot{M}O_2$ values recorded over the entire monitoring period, a method that has yielded reliable results across various fish species (Chabot et al. 2016; Andersson et al. 2020). Only $\dot{M}O_2$ values with an r^2 above 0.95 were included in the SMR calculations. Differences between MMR and SMR for each species were calculated as both absolute aerobic scope (AAS = MMR - SMR) and factorial aerobic scope (FAS = MMR / SMR), as these two metrics often provide complementary information (Halsey et al. 2018). Microbial respiration, though minor compared to fish $\dot{M}O_2$, was accounted for by assuming a linear change over time (Rodgers et al. 2016; Killen et al. 2021). Specifically, we measured microbial respiration in empty chambers immediately before and after each trial, taking the average of three $\dot{M}O_2$ measurements. To prevent excessive microbial buildup, we cleaned the system between trials with bleach, dried it with paper towels, and refilled it with water at the target salinity.

The videos of the behavioural trials were tracked using custom tracking software (ATracker by JWJ), which employs background subtraction methods to accurately determine the exact coordinates of the animals' centroid on a frame-by-frame basis. All tracking files were carefully inspected visually, and any tracking issues resolved through the manual tracking interface of ATracker. Afterwards, we processed the files, filled in missing frames, converted the positional data to millimetres, smoothed the trajectories, and for each frame calculated individuals' speed, heading, turning speed, and distance to the tank walls. Subsequently, we computed the following summary statistics for each trial: median speed (cm/s), proportion of time stationary (> 1 cm/s, based on plotting speed distribution curves), median turning speed (degrees/s), proportion of sharp turning (> 45 °/s), proportion of time near the edges of the arena, proportion of arena visited, and the coefficient of variation (CV) of proportion of arena visited. To account for spurious heading changes that may happen on a frame-by-frame basis at very low speeds, heading and turning speed were calculated on a second-by-second basis. For the measures of exploration, the arena was divided into equal 3 cm sections, creating a grid of 20 × 20 bins. We then calculated the proportion of arena explored as the number of bins containing tracking data divided by the total number of bins. The coefficient of variation of the explored area was calculated as the standard deviation of the tracking points across the bins divided by the mean, expressed as a percentage. For fish that spent more than 50% of the trial in the plants (n = 2), these measures were not calculated, as the measure would mainly reflect increased hiding behaviour rather than differences in movement tendencies.

Statistical analyses

To analyse differences in the four metabolic rates (MMR, SMR, FAS, and AAS) while accounting for fish mass, we used linear mixed models with species, sex, and salinity treatment as fixed effects, fish mass as a covariate, and lagoon as random effect. Likelihood ratio tests and Akaike information criteria (AIC) indicated that random intercept models (rather than random slope models) and those assuming homogeneity of slopes between mass and the categorical factors were preferable. We used the *lmer* function from the *lme4* package (Bates et al. 2015) to fit the mixed models and calculated *P* values with the *lmerTest* package (Kuznetsova et al. 2017). Additionally, we computed the marginal and conditional R^2 with the *MuMIn* package (Bartoń 2023). The marginal R^2 represents the variability explained solely by the fixed effects, while the conditional R^2 accounts for the variability explained jointly by both the fixed and random effects. Note that, instead of *F* statistics, we report the Sums of Squares (SS), which are closely related to η^2 (eta squared) and other measures of effect size (Masó et al. 2025). The four response variables (metabolic traits) and fish mass were $\log_{10}$-transformed in all analyses to linearise the relationships, and the model assumptions were verified using residual plots. To analyse the effects of the same sources of variation (salinity, species, sex, and fish mass) on the behavioural variables, we used a multivariate analysis of covariance (MANCOVA), followed by analyses of covariance (ANCOVAs) separately for each response variable (see e.g García-Berthou and Moreno-Amich 1993). Finally, we used correlation and redundancy analyses (RDA) to analyse the relationship between behaviour and metabolism. RDA is a constrained linear ordination method that combines principal component analysis and regression, allowing for the modelling of multivariate response data (Borcard et al. 2011; Legendre and Legendre 2012), and was performed using the *rda* function in the *vegan* package of R (Oksanen et al. 2024) to assess the relationship of behavioural traits with species and metabolic traits. Permutation tests with the *anova.cca* function were used in this case to test the significance of axes and the predictors. All analyses and figures were performed using R version 4.4.0 (R Core Team 2024) and relied on *ggplot2* (Wickham 2016) and *viridis* (Garnier et al. 2024) for visualisations.

Results

Metabolic traits

Except for FAS, the mixed models explained most of the variation in metabolic traits (65-80%), which was markedly dependent on fish mass (Table 1; Suppl. material 3: fig. S2). MMR and AAS (log-transformed) were strongly correlated for both species (Suppl. material 3: figs S3, S4), as MMR was much higher than SMR (Fig. 1, Suppl. material 3: fig. S2). After accounting for mass, the salinity treatment and its interaction with species were the most important sources of variation (see SS in Table1). Toothcarp, particularly females, showed overall higher SMR and MMR than mosquitofish after accounting for mass (Fig. 1; Suppl. material 3: fig. S2).

Table 1. Linear mixed models for four metabolic traits in mosquitofish and toothcarp across salinity treatments. Fixed effects included salinity, species, sex, and log_{10}-transformed fish mass; lagoon was included as a random effect. The response variables and mass were log_{10} transformed. Marginal and conditional R^2 are reported in parentheses (only marginal R^2 was available for FAS due to singularity). SS = Sum of Squares; *df* = degrees of freedom. Significant effects are bolded; *** indicates $P < 0.001$.

Source of variation	SMR (0.751, 0.798)			MMR (0.749, 0.795)			AAS (0.651, 0.690)			FAS (0.158)		
	SS	*df*	*P*	SS	*df*	*P*	SS	*df*	*P*	SS	*df*	*P*
Mass	3.35	1, 175	***	3.31	1, 161	***	3.45	1, 161	***	0.10	1, 162	0.361
Species	0.08	1, 174	***	0.07	1, 158	**0.001**	0.07	1, 148	**0.017**	0.01	1, 162	0.500
Sex	0.03	1, 174	0.052	0.01	1, 161	0.254	0.03	1, 159	0.155	0.08	1, 162	**0.010**
Salinity	0.27	1, 175	***	0.04	3, 161	0.101	0.03	3, 161	0.547	0.16	3, 162	**0.005**
Species × Salinity	0.07	3, 174	**0.010**	0.15	1, 161	***	0.16	1, 161	***	0.02	1, 162	0.184
Sex × Salinity	0.21	2, 174	***	0.03	3, 161	0.233	0.01	3, 161	0.793	0.09	3, 162	0.054

While toothcarp exhibited higher SMR at both the lowest and the highest salinity levels tested, mosquitofish showed decreasing SMR and MMR with increasing salinity. Sex or the salinity × species interaction was also significant for SMR and FAS (Table1): toothcarp females had higher SMR but lower FAS than males (Fig. 1). The random effect (lagoon) was generally significant ($P < 0.05$) but explained little additional information (always less than 5%).

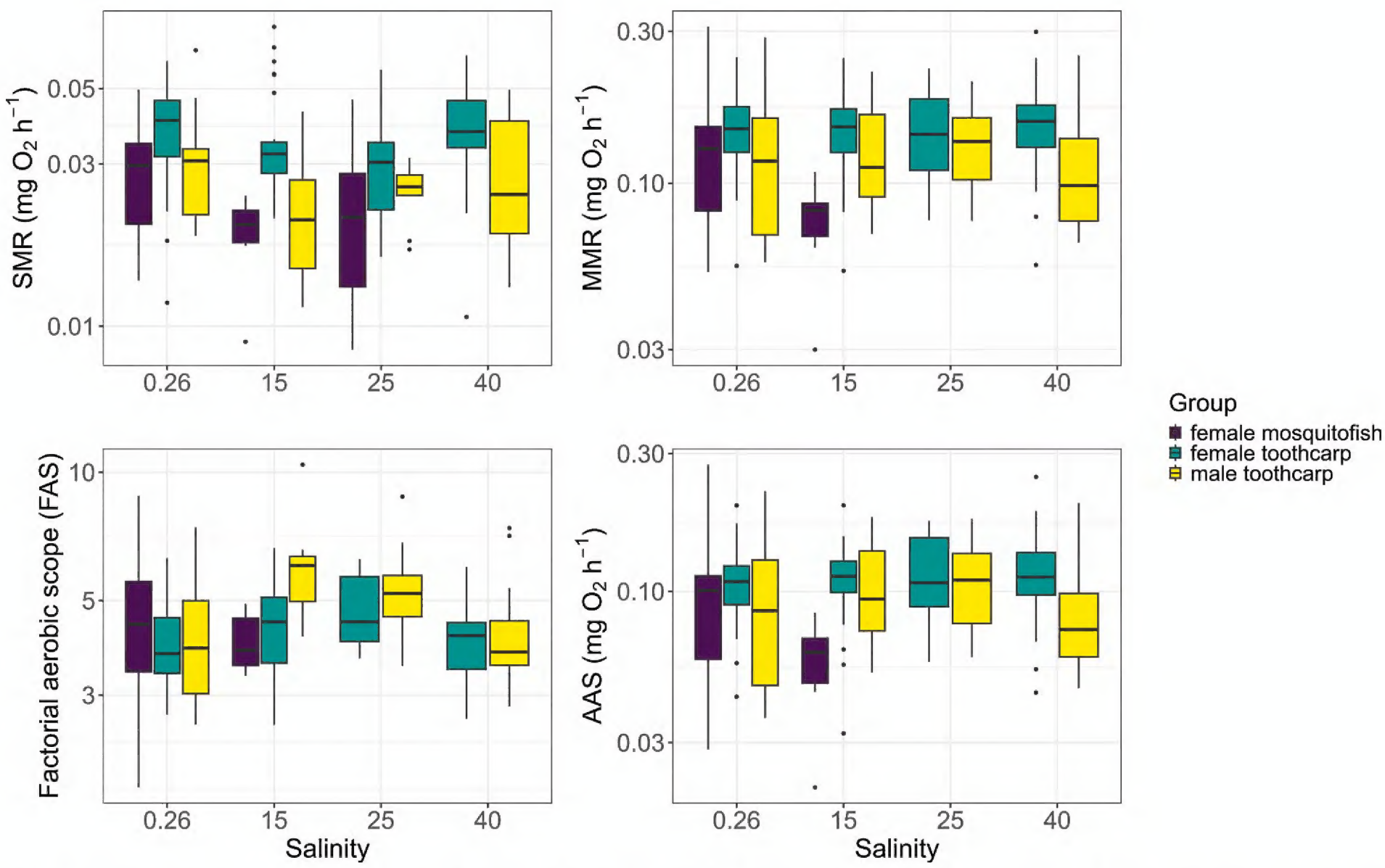

Figure 1. Box-plots of metabolic responses (SMR, MMR, FAS, and AAS) for female mosquitofish (purple), female toothcarp (green), and male toothcarp (yellow) across salinity treatments. Note the log_{10} scale on the *y*-axes. Boxes represent the first and third quartiles; lines indicate the medians; whiskers show the maximum and minimum values, excluding outliers (dots).

Behavioural movement variables

Overall, the ensemble of behavioural variables (which showed little inter-correlation, Suppl. material 3: figs S3, S4) displayed strong effects of species, salinity, and mass, with no clear interactions (Table 2). Follow-up ANCOVAs revealed that mosquitofish spent more time in a moving state and were more uniform in how they visited the different parts of the arena (lower CV of arena visited) than toothcarp (Fig. 2), while there were no major differences between the species for the other variables. In terms of salinity, there was no effect on the majority of behavioural variables (Table 2) except for the turning tendency of the fish, with both species exhibiting fewer sharp turns at higher salinity.

Table 2. Multivariate analysis of covariance (MANCOVA) of the seven behavioural variables using salinity, mass, species, and sex as predictors. Pillai's trace values, *F*-statistics, degrees of freedom (*df*), and significance levels (*P*) are presented for each source of variation. Adjusted $R^2 = 0.92$. *** indicates $P < 0.001$; significant results are bolded.

Source of variation	Pillai's trace	*F*	*Df*	*P*
Mass (log_{10} transformed)	0.334	3.013	7, 42	**0.012**
Species	0.603	9.111	7, 42	***
Sex	0.180	1.318	7, 42	0.266
Salinity	0.312	2.721	7, 42	**0.020**
Salinity × Species	0.085	0.558	7, 42	0.785
Salinity × Sex	0.083	0.540	7, 42	0.799

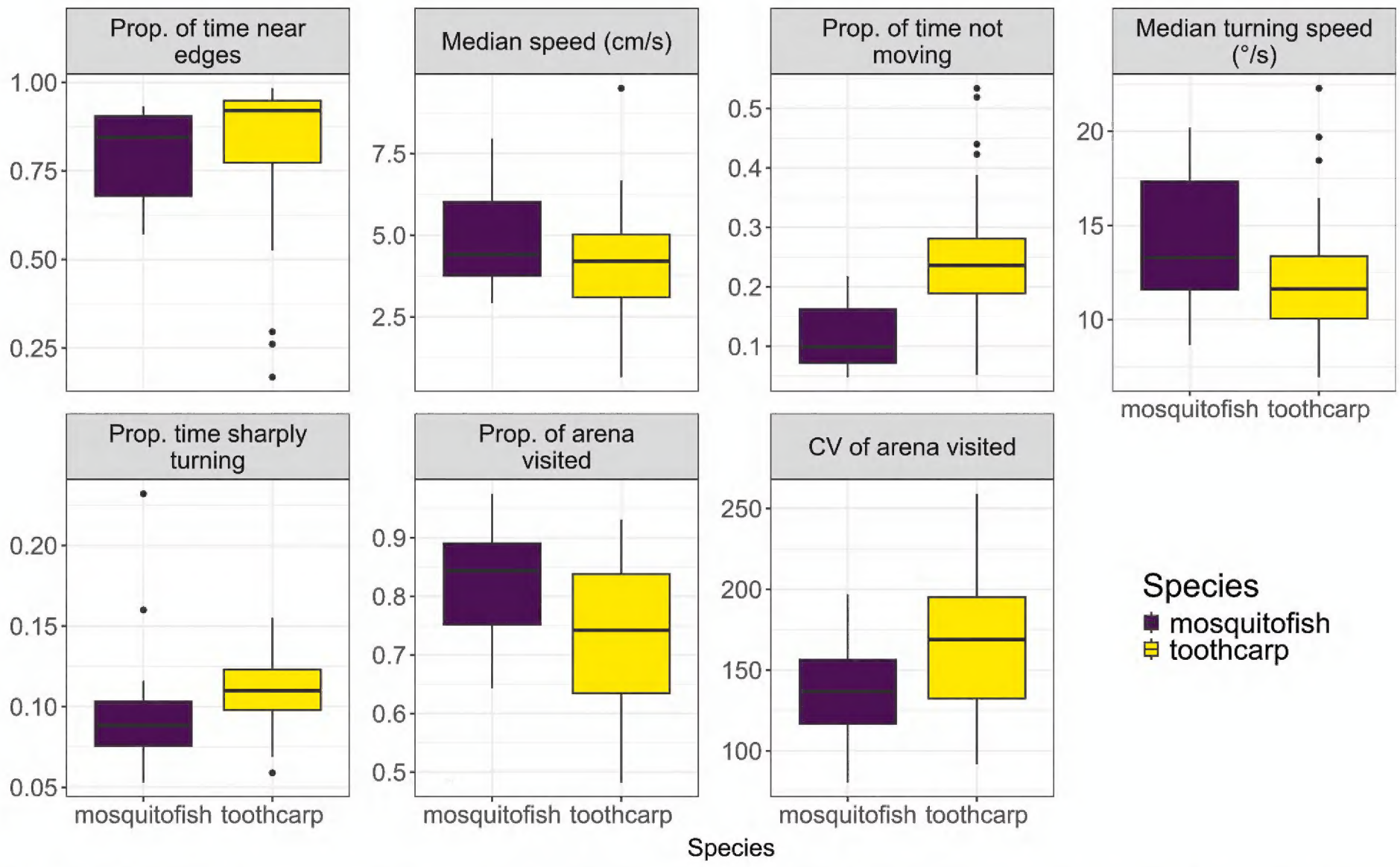

Figure 2. Box-plots of movement behavioural variables of mosquitofish (purple) and toothcarp from both sexes (yellow) pooled across salinity treatments. Metrics include the proportion of time near edges, median speed, proportion of time stationary, median turning speed, proportion of time sharply turning (> 45 °/s), proportion of arena visited, and the coefficient of variation (CV) of arena use.

Finally, mass impacted the median turning speed, the proportion of arena visited, and the time spent near the walls, with heavier fish across both species turning slower, visiting less of the arena, and swimming more near the tank walls (Suppl. material 3: figs S3, S4). Redundancy analyses (RDA) confirmed that species and fish mass were significant (P values < 0.05) for the ensemble of behavioural variables and summarised the differences between the two species well (Suppl. material 3: fig. S5).

Species explained 15.8% of the variation, with the main effect being that mosquitofish spent more time moving, had a higher median turning speed, and visited a larger proportion of the arena compared to toothcarp (Suppl. material 3: figs S2, S5).

Relationship between metabolism and behaviour

The RDA of behavioural variables as a function of metabolic rates was also performed separately for each species. In toothcarp, it explained 19.1% of the behavioural variation (Fig. 3), with SMR being the only significant predictor (P = 0.023). This effect was driven by a negative association between SMR and the proportion of the arena visited, and positive associations with both spatial variability (CV of arena) and time spent near the tank walls (Fig. 3, Suppl. material 3: fig. S3), indicating that individuals with higher baseline metabolic costs showed more restricted and edge-oriented behaviour.

In addition, FAS was significantly correlated with individual movement traits, being positively related to median speed and negatively related to the proportion of time spent stationary (Fig. 4, Suppl. material 3: fig. S3), suggesting that individuals with greater aerobic capacity tended to swim faster and remain active for longer periods. These metabolic–behaviour relationships in toothcarp were not attributable to differences in body mass (multiple regression, P = 0.63; Fig. 4). For mosquitofish, the RDA explained 21.6% of the behavioural variation (Suppl. material 3: fig. S4), but no single metabolic variable significantly explained behavioural differences when considering the ensemble of traits (Suppl. material 3: fig. S6). However, when examining individual correlations, median speed was the behavioural variable most strongly associated with metabolic performance. Specifically, it was positively related to MMR and, by extension, also to AAS (Suppl. material 3: fig. S4), indicating that individuals with higher maximum and aerobic scope capacities tended to swim faster. Despite this, the relationship between FAS and median speed was not significantly different between species (ANCOVA, P = 0.13; Fig. 4).

Our results collectively demonstrate clear species differences in behaviour, metabolism, and the interconnections between these characteristics. RDA and ANCOVAs showed that toothcarp were more restricted and edge-oriented in movement, while mosquitofish explored more broadly and spent more time moving. LMMs indicated that toothcarp had higher SMR and MMR across salinities, and RDAs and correlation analyses revealed stronger links between metabolism and behaviour in this species, particularly a negative association between SMR and space use. By contrast, mosquitofish exhibited a markedly lower AAS at higher salinities, weakly related to their behavioural movement, primarily between MMR and median swimming speed.

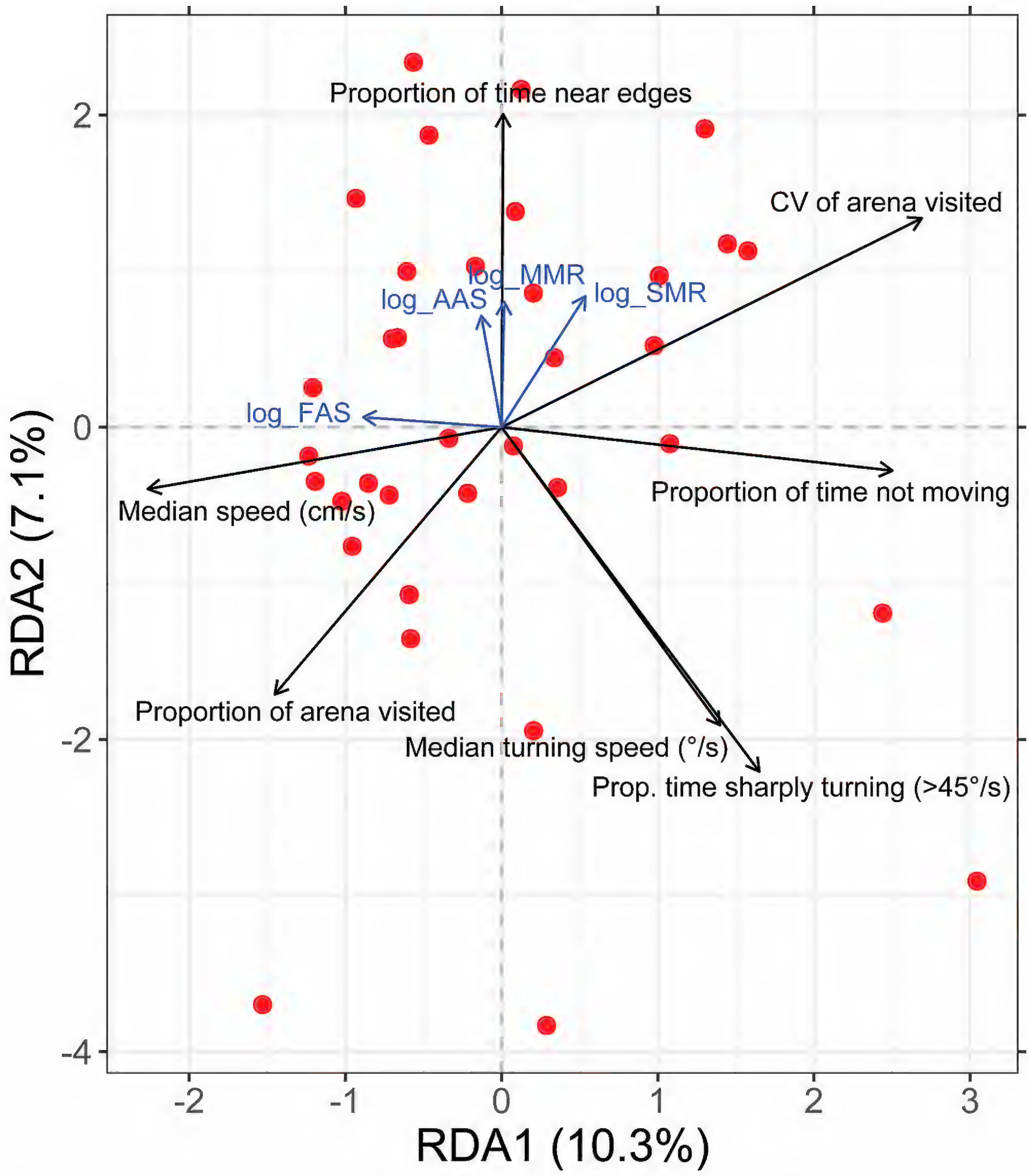

Figure 3. Redundancy analysis of the seven behavioural variables (black arrows) with metabolic traits (blue arrows) as constraints in toothcarp (see Suppl. material 3: fig. S6 for the plot for mosquitofish). Each red dot represents an individual.

Discussion

We investigated the physiological and behavioural responses of two competing fish species of marked ecological importance: the widely invasive eastern mosquitofish and the endangered, locally restricted Spanish toothcarp, under different experimental salinity conditions known to influence their interactions. Our results show

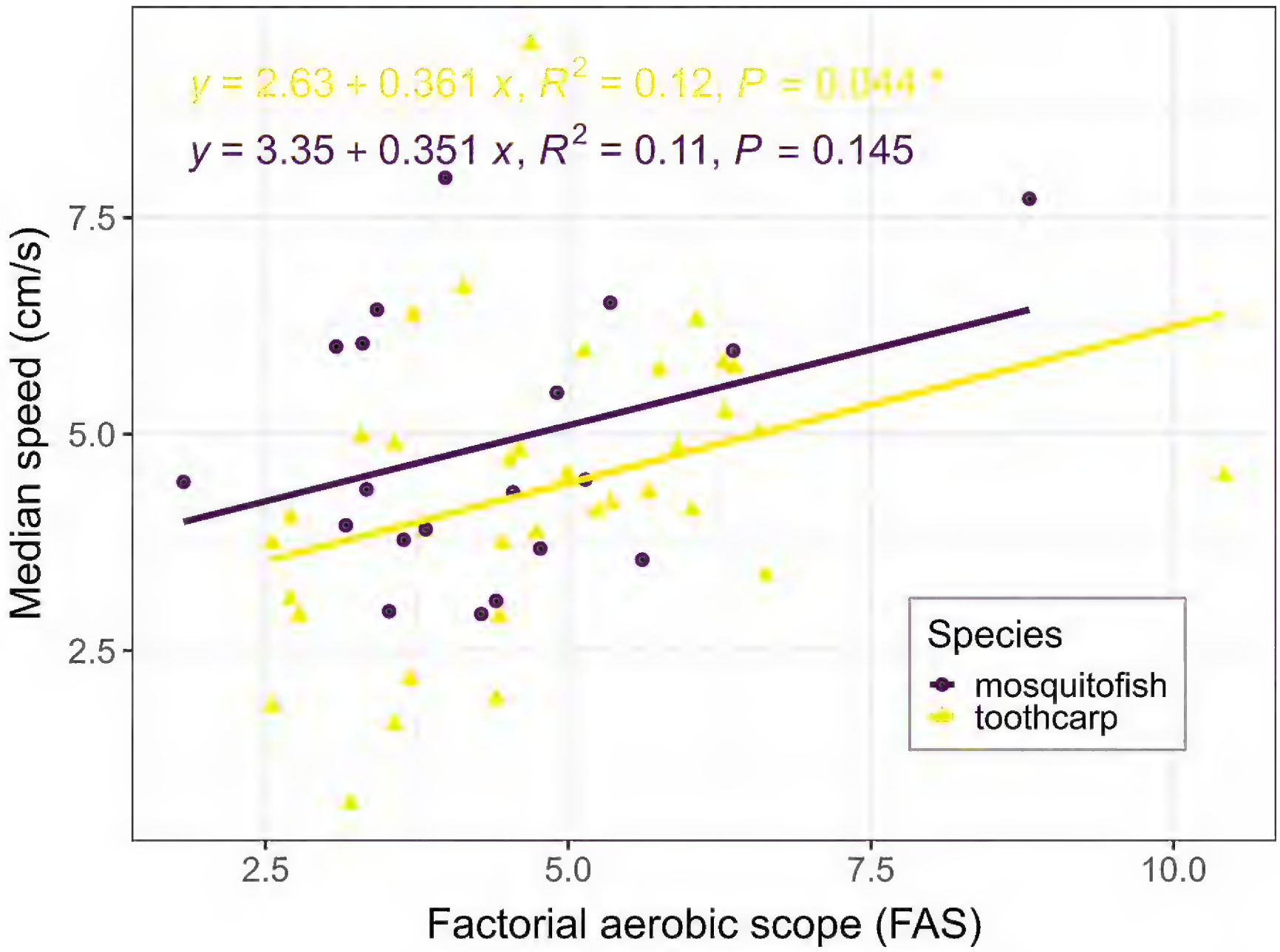

Figure 4. Relationship between the median speed and factorial aerobic scope (FAS) for mosquitofish (purple) and toothcarp from both sexes (yellow) pooled over two salinity treatments. * Indicates a significant relationship.

that toothcarp maintained consistent metabolic performance across different salinities and displayed links between metabolism and behaviour, with SMR negatively associated with movement. Conversely, mosquitofish exhibited lower aerobic capacity at moderate salinity, but with movement patterns being more independent of their metabolic traits. The two species also differed in their movement tendencies, with toothcarp demonstrating more restricted and clustered movement compared to mosquitofish. Overall, the two species differed not only in their metabolic and behavioural traits but also in the relationships between them.

The metabolic responses of mosquitofish and toothcarp confirmed contrasting salinity tolerances, as previously hypothesised (Alcaraz et al. 2008a). SMR was lower at 15 than at 0.26 in both species, likely because 15 is closer to the iso-osmotic point and involves reduced osmoregulatory costs (Ern et al. 2014; Brennan et al. 2016; Nortsog et al. 2022). This effect was more evident in mosquitofish, highlighting differential responses to salinity between the two species (Ern et al. 2014). These differences were even more pronounced in terms of MMR and AAS. Previous research has indicated that toothcarp may have a slightly higher MMR than mosquitofish (Rubio-Gracia et al. 2020). Here, we show that these differences increase with salinity: toothcarp maintained stable MMR and AAS while mosquitofish exhibited lower values at 15. These differences may arise from several factors. On the one hand, in toothcarp, stability across salinities aligns with the low osmoregulatory costs associated with their euryhaline nature, as observed in other brackish-water cyprinodontiforms (Plaut 2000; Nordlie 2006; Oliva-Paterna et al.

2007; Brennan et al. 2016). On the other hand, in mosquitofish, lower AAS at 15 indicates restricted aerobic capacity even at moderate salinities. AAS was expected to be lower at higher salinities due to increased osmoregulatory costs (Nordlie and Mirandi 1996); however, the lower SMR at 15 suggests otherwise (Ern et al. 2014). Hence, the AAS observed here results from a markedly low MMR, indicating alternative constraints beyond osmoregulatory maintenance costs. One potential explanation is that, at higher salinities, fish gills reduce permeability to minimise water loss, thereby limiting oxygen uptake and constraining MMR (Plaut 2000; Sardella and Brauner 2007; Christensen et al. 2019). However, this idea conflicts with findings in the closely related western mosquitofish (*Gambusia affinis*), which exhibits high gill plasticity and sustains metabolic function under elevated salinities (Uliano et al. 2010). Whether eastern mosquitofish share this trait remains uncertain, although our results suggest otherwise. Further research should investigate gill permeability and plasticity among different populations of eastern mosquitofish, especially given their worldwide invasive distribution, including high-salinity environments (Ruiz-Navarro et al. 2011; Monti et al. 2021), high genetic variability (Congdon 1994; Vera et al. 2016), and the potential for population-level adaptation in these traits, as seen in other widespread freshwater fish species (Christensen et al. 2017; Christensen et al. 2019).

In terms of movement tendencies, despite sharing ecological similarities as shoaling species that often co-occur and compete for resources (Caiola and de Sostoa 2005; Alcaraz and García-Berthou 2007; Ruiz-Navarro et al. 2013), we observed notable differences in space use. Mosquitofish moved more continuously, both in terms of speed and turning, and explored a larger part of the arena and in a more uniform way, as indicated by a lower CV. These patterns align with increased swimming efficiency, supported by their streamlined body shape and higher optimal swimming speeds (Alemadi and Jenkins 2008; Srean et al. 2017; Rubio-Gracia et al. 2020). By contrast, the stationary movement of toothcarp indicates poor swimming efficiency, linked to their high cost of transport (COT), which demands significant energy expenditure (Rubio-Gracia et al. 2020). To conserve energy, they may rely on intermittent locomotion, characterised by bursts of activity followed by recovery pauses (Kramer and McLaughlin 2001). This approach suits species with a limited capacity for sustained movement, as it maximises muscle output, improves perception and risk assessment (Kramer and McLaughlin 2001; Wilson and Godin 2010), and may be related to their greater use of refuges (Magellan and García-Berthou 2016). However, it results in patchy distributions, which could explain their restricted home ranges and vulnerability to habitat fragmentation (Alcaraz et al. 2008b; Gonzalez et al. 2018). Conversely, the broader and more uniform use of space by mosquitofish reflects a more efficient exploratory strategy, linked to invasion success in other mosquitofish species (Cote et al. 2010), especially in slow-flow environments (Srean et al. 2017; Gatto and Trexler 2020).

Regarding salinity effects on behaviour, the lack of species differences across treatments was unexpected, especially given the distinct responses in AAS. If salinity imposes physiological constraints on mosquitofish, one might expect reduced speed or time spent moving. For instance, fathead minnows (*Pimephales promelas*) move less with increasing salinity (Hoover et al. 2013). Similarly, mosquitofish reduce prey capture at 15 and 25 when competing with a native toothcarp (Alcaraz et al. 2008a). It is possible that 15, although metabolically challenging, still allows for normal

locomotion while limiting more demanding behaviours. Consequently, at higher salinities, the effects may be more pronounced, potentially hindering locomotion severely (Pirroni et al. 2024). Salinity, however, did affect both species in other aspects of movement, with lower turning rates at 15, potentially suggesting lower exploratory behaviour (Lorenz et al. 2016; Leite et al. 2022) or more directed behaviour.

The relationship between metabolic traits and movement revealed distinct patterns for each species. In toothcarp, movement was influenced by both baseline energy demands (i.e. SMR) and the energy available beyond maintenance (i.e. FAS). SMR correlated negatively with the arena visited and positively with the CV in space use, suggesting that SMR may be a limiting factor to movement due to elevated COT, which results in intermittent locomotion (Kramer and McLaughlin 2001; Whitney et al. 2016; Rubio-Gracia et al. 2020). Accordingly, Killen (2014) observed in common minnows (*Phoxinus phoxinus*) that differences in SMR lead to differences in movement, where individuals with higher SMR are less active and prefer cooler water allowing individuals to reduce maintenance costs. Similarly, in the toothcarp studied here, individuals with higher SMR moved less, whereas those with lower SMR moved more and more broadly. Therefore, differences in SMR may drive interindividual distinct movement tendencies and influence space use and access to resources in this species (see Masó et al. 2025). Additionally, FAS positively correlated with speed and activity, suggesting that individuals with a greater aerobic capacity (i.e. MMR) are better equipped to sustain movement for longer (Halsey et al. 2018).

Mosquitofish showed weak associations between metabolic and movement traits, with only median speed correlating with MMR. This suggests that routine speed depends more on their metabolic ceiling than on baseline costs, allowing them to reach higher speeds than toothcarp. It is important to note, however, that the behaviours assessed involved free, low-intensity movement and may not fully reflect the energetic constraints of more demanding activities (Careau et al. 2008; Metcalfe et al. 2016). In mosquitofish, energetically costly actions such as female aggression have been linked to AAS rather than SMR and can be sustained even under physiological stress, such as late-stage pregnancy (Seebacher et al. 2013). This ability to operate near aerobic limits may help explain their consistent movement under moderate salinity, despite a reduced aerobic scope. More broadly, this plasticity in energy allocation may account for the weak relationship between metabolism and behaviour in our findings. Polverino et al. (2018) provide a case in point, demonstrating that these relationships can vary across mosquitofish populations depending on the ecological context, thereby highlighting the flexibility of trait integration in this species.

Our findings provide a valuable foundation for better understanding this mosquitofish–toothcarp system; however, several shortcomings should be acknowledged. Mosquitofish were tested at the lower salinities (0.26 and 15) in the respirometry assays, and behavioural trials for both species were conducted exclusively at these two salinity treatments. In the case of mosquitofish, only females were included because males from our site exhibited an extremely low tolerance to salinity. Trials were also conducted in two separate experimental phases, across seasons and populations, which limits direct comparability but allows for consistent conditions between species. Future work could examine movement responses under higher salinities and better assess the tolerance of mosquitofish females and males across a wider salinity range, as sex-specific traits may influence invasion outcomes in this

species (Michelangeli et al. 2020). Furthermore, comparing behavioural responses across salinity gradients in both solitary and social contexts would shed light on the broader repercussions of salinity stress.

In summary, salinity changes, such as those occurring in coastal wetlands, can alter competitive dynamics between INS and native species by modifying aerobic metabolism and functional capacity. In our study, toothcarp maintained stable metabolism across all treatments, whereas mosquitofish showed lower aerobic capacity at moderate salinity, driven by a lower MMR. This reduction in aerobic ceiling indicates that salinity can constrain performance without increasing osmoregulatory costs, with potential consequences for other INS entering these ecosystems. The two species also exhibited contrasting movement patterns, reflecting differences in dispersal potential and microhabitat use between INS and native species that co-occur (Rehage et al. 2005; Cote et al. 2010; Damas-Moreira et al. 2019). Toothcarp activity was constrained by baseline metabolism, whereas mosquitofish maintained similar activity despite reduced aerobic capacity. This mismatch, which was not observed in toothcarp, highlights the importance of integrating metabolic and behavioural traits to understand contrasting responses of invasive and native taxa and forecast invasion outcomes under environmental change, in line with recent calls to link physiology and behaviour more explicitly (Debaere et al. 2024). Further research in this field is essential, as trait-based approaches help predict species responses to abiotic stressors (Clark et al. 2013) and provide a basis for assessing INS performance relative to native taxa across environmental stressors (Marras et al. 2015; Taylor and Dunn 2018; Behrens et al. 2020; Christensen et al. 2021; Backström and Winkelmann 2022; Vivó-Pons et al. 2024).

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Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

The capture of wild animals, animal care, and the experimental protocols were conducted in compliance with the Autonomous Government of Catalonia (Ref. CEEAH5913_OH003), the Commission of Animal Experimentation of the Autonomous University of Barcelona (Ref. 3782-CEEA-UAB), and the authorities of Montgrí, Illes Medes i Baix Ter Natural Park.

Use of AI

No use of AI was reported.

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Author contributions

The five authors contributed equally to this work. AVG, FCR and JJ designed the study; FCR and JJ performed the field and laboratory work; EGB conducted the statistical analyses; MBM led the writing; all authors edited the manuscript.

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Data availability

All the data that support the findings of this study are publicly available on Figshare: https://doi.org/10.6084/m9.figshare.30666989.

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Supplementary material 1

Data

Authors: Michael Butler-Margalef, Francesc Rubio-Gracia, Emili García-Berthou, Jolle W. Jolles, Anna Vila-Gispert

Data type: docx

Explanation note: Total mass and metabolic traits of Spanish toothcarp and mosquitofish by salinity treatment (practical salinity scale). Means, standard errors (SE) and ranges (minimum and maximum) are shown.

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Link: https://doi.org/10.3897/neobiota.105.170980.suppl1

Supplementary material 2

Checklist

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Data type: docx

Explanation note: Checklist of intermittent-flow respirometry setup for metabolic rate measurements in mosquitofish and toothcarp, reported according to the guidelines of Killen et al. (2021).

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Link: https://doi.org/10.3897/neobiota.105.170980.suppl2

Supplementary material 3

Supplementary figures

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Data type: pdf

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Link: https://doi.org/10.3897/neobiota.105.170980.suppl3